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PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Ts'o et al.

Application No. 09/888,164

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Art Unit: Unassigned

Examiner: Unassigned

For: LIGANDS TO ENHANCE CELLULAR UPTAKE OF BIOMOLECULES

AMENDMENTS TO SPECIFICATION AND CLAIMS MADE VIA PRELIMINARY AMENDMENT

Amendments to specification:

Amendments to the "CROSS REFERENCES" section on page 1, lines 4-5:

CROSS-REFERENCE TO RELATED PATENT APPLICATIONS

This patent application is a continuation of U.S. patent application no. 09/282,455 filed on March 31, 1999, abandoned, which is a continuation-in-part of U.S. patent application no. 08/755,062 filed November 22, 1996, now US. Patent No. 5,994,517, which claims priority to U.S. provisional patent application no. 60/007,480 filed November 22, 1995. [This is a continuation-in-part of U.S. Serial No. 08/755,062, filed November 22, 1996.]

Amendments to the paragraph starting on page 11, line 26, and ending on page 12, line 1:

Figure 2 shows the structures of neoglycopeptide YEE(ahGalNAc)$_3$ **(5)** **(Figure 2a)**; oligo-MP $U^{m}p\underline{T}_7$ **(6)**, and 5'-ethylenediamine capped $U^{m}p\underline{T}_7$ **(6b)** **(Figure 2b)**; Structure of the Tracer, 3' conjugate **(Figure 2c)**; Reaction scheme for the automated synthesis of SEQ ID NO.:32 with 5'-thiol modifier **(Figure 2d)**; and Reaction scheme for the synthesis of **1c** comprising SEQ ID NO.:32 **(Figure 2e)**.

Amendments to the paragraph on page 12, lines 10-11:

Figure 5 illustrates the structures of the [^{35}S]3′-End Labeled hepatitis B virus (HBV) neoglycoconjugates (NG1 is SEQ ID NO.:27; NG2 is SEQ ID NO.:28; NG3 is SEQ ID NO.:29; NG4 is SEQ ID NO.:30).

Amendments to page 22, line 33:

<u>TGCTCATGGTGCACGGTCTACGA</u> [TFCTCATGGTGCACGGTCTACGA] (SEQ ID NO.: 8)

In re Appln. of Ts'o et al.
Application No. 09/888,164

Amendments to page 40, Table 5:

Table 5. Oligonucleotide Alternating Methylphosphonate Analogs.

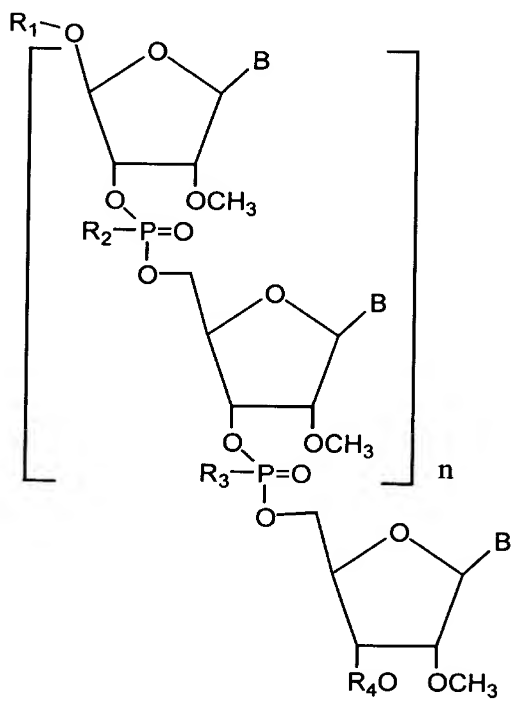
Sequence

1 (n=7) ApGp̲UpCp̲ApGp̲UpCp̲ApGp̲UpCp̲ApGp̲U (SEQ ID NO.:24)

2 (n=7) GpUp̲UpCp̲UpCp̲CpAp̲UpGp̲UpUp̲CpAp̲G (SEQ ID NO.:25)

3 (n=10) UpUp̲UpAp̲UpAp̲ApGp̲GpGp̲UpCp̲GpAp̲UpGp̲UpCp̲CpAp̲U (SEQ ID NO.:26)

where p: phosphodiester linkage
p̲: methylphosphonate linkage
ps: phosphorothioate linkage



Oligonucleotide	R_1	R_2	R_3	R_4
a	H	O^-	CH_3	3'-conjugate
b	C6-thiol-ps	O^-	CH_3	3'-conjugate
c	5'-conjugate	O^-	CH_3	3'-conjugate
d	Ligand-SMCC-AET	O^-	CH_3	H
e	EDA	O^-	CH_3	H

where Ligand: YEE(ah-GalNAc)$_3$
5'-conjugate: YEE(ah-GalNAc)$_3$-SMCC-S(CH_2)$_6$-ps linkage (Figure 3)
3'-conjugate: Tracer Unit (Figure 9)
EDA: ethylenediamine

Amendments to the paragraph starting on page 47, lines 2 21:

The purified disulfide-containing oligomers were then used in conjugation with SMCC-YEE(ah-GalNAc)$_3$ similarly as described in Example 2. Most conjugation reactions were performed by using 1.5-2 equivalents of SMCC-YEE(ah-GalNAc)$_3$ to the thiol oligomers. These resulted in quantitative conjugation of the oligomers in all of the reactions performed. Excess ligand and buffer salts were easily removed by a G-25 column, eluting with 20% ethanol, to give highly pure conjugates. Conjugation reactions were also performed using excess amount of thiol oligomers instead, e.g., 1.5 equivalent of the thiol oligomers to the ligand. In these cases, all of the ligands were consumed in the reactions and the remaining excess amount of thiol-oligomers were removed by preparative reversed phase high pressure liquid chromatagraphy (HPLC). Following are the sequences of four oligodeoxyribonucleoside phosphorothioate A-L-P conjugates synthesized by the above method (**Figure 5**). NG1: YEE(ahGalNAc)$_3$-SMCC-5'GTTCTCCATGTTCAG3' <u>(SEQ ID NO.:27)</u>, which targeted the HBV sa-gene, NG2: YEE(ahGalNAc)$_3$-SMCC-5'TTTATAAGGGTCGATGTCCAT3' <u>(SEQ ID NO.:28)</u>, which targeted the HBV c-gene, NG3: YEE(ahGalNAc)$_3$-SMCC-5'AAAGCCACCCAAGGCA3' <u>(SEQ ID NO.:29)</u>, which targeted the HBV e-site, and the random controls, NG4: YEE(ahGalNAc)$_3$-SMCC-5'TGAGCTATGCACATTCAGATTT3' <u>(SEQ ID NO.:30)</u>, and NG5: YEE(ahGalNAc)$_3$-SMCC- 5'TCCAATTAGATCAG3' <u>(SEQ ID NO.:31)</u>.

Amendments to the paragraph starting on page 55, line 9, through page 56, line 6:

The above examples illustrate that OMNP's can be conjugated to the hepatic specific ligand YEE(ah GalNAc)$_3$ to yield a homogeneous and defined neoglycoconjugate. Furthermore, this neoglycoconjugate is taken up by hepatoma-derived cells (Hep G2) specifically and at an enhanced rate in vitro. The above results have been extended to consider oligonucleotides with other nuclease resistant backbone modifications, such as phosphorothioates (ps) oligomers comprised of 2'Omethyl ribose moieties and alternating phospho-diester/methylphosphonate linkages (2'Ome-po/mp). The experimental methods were identical to those utilized in Examples 4 and 5. Results of these experiments were very similar to those observed with the OMNP containing neoglyco-conjugates. Neoglycoconjugate containing phosphorothiate oligomers were synthesized according to Conjugate Method 2. YEE (ahGalNAc)$_3$-SMCC-ps $^{5'}$GTTCTCCATGTTCAG$^{3'}$ (NG-1)

(SEQ ID NO.:27) was labeled with ^{35}S using the 3′-end labeling method described in Conjugation Method 2 displayed a linear uptake to the extent of 17.25 pmoles/10^6 cells at 24 hours. In contrast the corresponding unconjugated oligomer ps $^{5'}$GTTCTCCATGTTCAG$^{3'}$ (SEQ ID NO.:27) was taken up by Hep G2 cells at a diminished rate, reaching 1.01 pmoles/10^6 cells at 24 hours. In a similar fashion, neoglyco-conjugates containing 2' OMe alternating po/mp oligomers (YEE(ahGalNAc)$_3$-SMCC-2'OMe $^{5'}AG_pUC_pAG_pUC_pAG_pUC_pAG_pU^{3'}$) (SEQ ID NO.:32) displayed a linear uptake to the extent of 24.3 pmoles/10^6 cells at 24 hours. The corresponding unconjugated oligomer (2'OMe $^{5'}AG_pUC_pAG_pUC_pAG_pUC_pAG_pU^{3'}$) (SEQ ID NO.:32) displayed minimal uptake of less than 1 pmole/10^6 cells at all time points assayed. All oligomers and neoglycoconjugates were stable in cell culture media up to 24 hours. These results illustrate the delivery utility of the unique ligand-linker complex and give us a platform to expand this system to the delivery of other therapeutic agents.

Amendments to the paragraph starting on page 56, line 27, through page 57, line 16:

The cellular uptake experiments described utilizing ^{32}P-labeled oligo-mp conjugates were extended to examine the cellular association of neoglycoconjugates comprised of neoglycopeptide **5** and oligomers of other nuclease resistant backbones, most notably ps and 2'OMe po/mp, with Hep G2 cells. Neoglycoconjugates containing phosphorothioate oligomer, YEE(ahGalNAc)$_3$-SMCC-ps $^{5'}$GTTCTCCATGTTCAG$^{3'}$ (NG-1) (SEQ ID NO.:27) was labeled using Conjugation Method 2, which displayed linear uptake to the extent of 17.25 pmoles/10^6 cells at 24 hours. In contrast, the corresponding unconjugated oligomer ps $^{5'}$GTTCTCCATGTTCA-G$^{3'}$ (SEQ ID NO.:27) was taken up by Hep G2 cells at a diminished rate, reaching 1.01 pmoles/10^6 cells at 24 hours. In a similar fashion, neoglyco-conjugates containing 2'OMe alternating po/mp oligomers (YEE(ahGalNAc)$_3$-SMCC-2'OMe $^{5'}AG_pUC_pAG_pUC_pAG_pUC_pAG_pU^{3'}$) (SEQ ID NO.:32) displayed a linear uptake to the extent of 28.52 pmoles/10^6 cells at 24 hours (**Figure 9**; Table 6). The corresponding unconjugated oligomer (2'OMe $^{5'}AG_pUC_pAG_pUC_pAG_pUC_pAG_pU^{3'}$) (SEQ ID NO.:32) displayed minimal uptake of less than 1 pmole/10^6 cells. These results illustrate the delivery utility of the unique ligand-linker complex and allow a platform to expand this system to the delivery of other therapeutic agents.

Amendments to Table 6 on page 59:

TABLE 6-Uptake of conjugated YEE(ah-GAlNac)$_3$-SMCC-AET-2'O-Me $^{5'}AG_PUC_PAG_PUC_PAG_PUC_PAG_PU^{3'}$ <u>(SEQ ID NO.:24)</u> (1d) and EDA-2'-O-Me- $^{5'}AG_PUC_PAG_PUC_PAG_PUC_PAG_PU^{3'}$ <u>(SEQ ID NO.:32)</u> (1e) by Hep 2G 2.2.15 cells in culture (pmoles/10^6 cells)

OLIGOMER	1 HOUR	2 HOURS	3 HOURS	24 HOURS
1d	3.63	7.71	14.16	28.52
1e	0.277	0.305	0.400	0.450

Amendments to Table 7 on page 59:

TABLE 7-Uptake of YEE(ah-GAlNac)$_3$-SMCC-S(CH$_2$)$_6$-*ps*- 2'O-Me- $^{5'}AG_PUC_PAG_PUC_PAG_PUC_PAG_PU^{3'}$ <u>(SEQ ID NO.:32)</u> –U*Dt*$^{3'-3'}$ (dt-T)-^{32}P-EDA (1c) by Hep G2 2.2.15 cells in culture (pmoles/10^6 cells)

OLIGOMER	4 HOURS	8 HOURS	12 HOURS	16 HOURS	24 HOURS
1c	9.44	18.60	22.05	24.92	28.97

Amendments to the paragraph on page 64, lines 4-22:

Male CD-1 mice were injected as described in Example 9 with 30 pmoles of the neoglycoconjugate YEE(ahGalNAc)$_3$-SMCC-ps-(TTTATAAGGGTCGATGTCCAT)-$^{\{35S\}}$(psA)$_n$ <u>(SEQ ID NO.:28)</u> labeled utilizing the 3'-end labeling method as decribed in Conjugation Method 2. For comparison, a conjugate which lacks the three terminal GalNAc residues, YEE(ah)$_3$-SMCC-ps-(TTTATAAGGGTCGATGTCCAT)-(psA)$_n$ $^{\{35S\}}$ <u>(SEQ ID NO.:28)</u> was also synthesized. This sugarless conjugate served as a control for the study of ligand (GalNAc)-specific uptake in mice. Experimental results were very similar to those observed in Example 10. The conjugate containing the terminal sugar residues associated to the greatest extent with the liver, reaching a value of 46.19 % of the injected dose 15 minutes post-injection. The ranking of total radioactivity in the other tissues measured at 15 minutes post-injection was, in decreasing order: muscle > blood > kidney >spleen. The peak value of radioactivity for the urine was 4.51% of the injected dose and was reached after 15 minutes. The amount of radioactivity associated with the kidneys and blood decreased over time.

Amendments to the paragraph on page 70, lines 18-28:

A tritium labeled 12 mer (d-Tp*TCCTCCTGCGG) (SEQ ID NO.:33) consisting of all methylphosphonate backbone except the last 5' terminal phosphodiester linkage was injected i.v. in a single dose in mice. Organs were collected in 2, 5, 10, 30, 60 and 120 minutes after drug administration. The data shows that the radioactivity was not allocated in liver, lung, muscle or spleen, and was rapidly disappearing from the plasma into the kidney and urine. The HPLC study showed that the intact 12-mer was metabolized to 11-mer via enzymatic cleavage of the terminal nucleotide and both were eliminated rapidly into the urine after i.v. injection. Thus, the results reported herein agree well with the results obtained earlier, demonstrating the importance of the GalNAc terminal in directing the uptake of oligomer conjugate into liver.

Amendments to the paragraph on page 75, lines 9-24:

Methods: The three therapeutic neoglycoconjugates utilized in this study were synthesized by conjugation of the following ps-oligomers, previously shown to inhibit HBV replication in vitro (Korba and Gerin, 1995, supra), to the liver specific ligand YEE(ahGalNAc)$_3$: (1) 5'GTTCTCCATGTTCAG3' (SEQ ID NO.:27) which targets the translation initiation site of the surface antigen gene (sa-gene), (2) 5'TTTATAAGGGTCGATGTCCAT3' (SEQ ID NO.:28) which targets the translational initiation site of the core gene (c-gene) and overlaps the HBV polyadenylation site and (3) 5'AAAGCCACCCAAGGCA3' (SEQ ID NO.:29) which targets the unpaired loop of the encapsidation site of the HBV pregenome (e-site). The base sequence used to synthesize the oligomers for this study was a HBV subtype ayw (Galibert, *et.al.*, (1979), *Nature* (London), 281:646-650), the same subtype expressed in vitro by HepG2 2.2.15 (Acs et al., (1987), *Proc. Natl. Acad. Sci.*, 84:4641-4644. In addition, two additional ps-oligomers, which are non-complementary to the HBV genome, NG4: $^{5'}$TGAGCTATGCACATTCAGATTT$^{3'}$ (SEQ ID NO.:30) and NG5: $^{5'}$TCCAATTAGATCAG$^{3'}$ (SEQ ID NO.:31), were prepared as controls to assay for non-specific effects of the ps-neoglycoconjugates.

Amendments to claims:

1. (Amended) A construct comprising a homogeneous conjugate of formula A-L-P, wherein

A represents a hepatic ligand that specifically binds to a hepatic receptor, thereby facilitating the entrance of said conjugate into cells having said receptor;

L represents a bifunctional linker that is covalently linked to A in a regiospecific manner to form A-L; A-L is covalently linked to P in a regiospecific manner to form A-L-P;

P represents a biologically stable oligomer that binds to a hepatic pathogen, wherein P is released from the conjugate following hydrolysis or reduction of at least one specific biochemical linkage, and contains internucleotide linkages resistant to enzymatic hydrolysis or biodegradation upon release from the conjugate.

4. (Amended) The construct of claim [3] 1, wherein said pathogen is a hepatic virus.

5. (Amended) The construct of claim [3] 1, wherein said pathogen is a liver parasite.

6. (Amended) The construct of claim 4, wherein said hepatic virus is a hepatitis virus.

8. (Amended) The construct of claim [7] 4, wherein said oligomer binds to a surface antigen of said hepatic virus.

9. (Amended) The construct of claim [7] 4, wherein said oligomer binds to a core antigen of said hepatic virus.

10. (Amended) The construct of claim [7] 4, wherein said oligomer binds to an encapsidation sequence of said hepatic virus.

13. (Amended) The construct of claim 5, wherein said liver parasite is plasmodium for malaria.

17. (Amended) The construct of claim 6, wherein said oligomer comprises [comprising] a sequence selected from the group consisting of GTTCTCCATGTTCAG (SEQ ID NO.: 27), TTTATAAGGGTCGATGTCCAT (SEQ ID NO.: 28), and AAAGCCACCCAAGGCA (SEQ ID NO.: 29).

18. (Amended) The construct of claim 2, wherein said oligomer [further] comprises deoxyribode methylphosphonate internucleotide linkages.

22. (Amended) The construct of claim 2, wherein said oligomer [further] comprises a combination of deoxyribose methylphosphonate/phosphodiester internucleotide linkages.

69. (Amended) The pharmaceutical composition of claim 68 wherein said oligomer comprises a sequence selected from the group consisting of $^{5'}$GTTCTCCATGTTCAG$^{3'}$ (SEQ ID NO.: 27), $^{5'}$TTTATAAGGGTCGATGTCCAT$^{3'}$ (SEQ ID NO.: 28), and $^{5'}$AAAGCCACCCAAGGCA$^{3'}$(SEQ ID NO.: 29).

71. (Amended) The pharmaceutical composition of claim 70 wherein said construct is selected from the group consisting of YEE(ahGalNAc)$_3$ – SMCC - $^{5'}$GTTCTCCATGTTCAG$^{3'}$ (SEQ ID NO.: 27), YEE(ahGalNAc)$_3$ - SMCC - $^{5'}$TTTATAAGGGTCGATGTCCAT$^{3'}$ (SEQ ID NO.: 28), and YEE(ahGalNAc)$_3$ - SMCC- $^{5'}$AAAGCCACCCAAGGCA$^{3'}$ (SEQ ID NO.: 29).

72. (New) The construct of claim 1, wherein the A-L moiety of said construct is YEE(ah-GalNAc)$_3$-SMCC.

73. (New) The construct of claim 1, wherein said construct is selected from the group consisting of YEE(ahGalNAc)$_3$ – SMCC - $^{5'}$GTTCTCCATGTTCAG$^{3'}$ (SEQ ID NO.: 27), YEE(ahGalNAc)$_3$ - SMCC - $^{5'}$TTTATAAGGGTCGATGTCCAT$^{3'}$ (SEQ ID NO.: 28), and YEE(ahGalNAc)$_3$ - SMCC- $^{5'}$AAAGCCACCCAAGGCA$^{3'}$ (SEQ ID NO.: 29).